LACK OF INFLUENCE OF MITOCHONDRIAL GENETICAL INFORMATION ON THE MULTIPLICATION OF HERPES SIMPLEX VIRUS IN HELA CELLS[1]

K. Radsak

Hygiene Institute of the University of Marburg

D-355 Marburg, BRD, Pilgrimstein 2

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Summary Mitochondrial DNA synthesis in HeLa cells is inhibited by 0.2 µg ethidium bromide/ml whereas nuclear DNA synthesis is essentially unimpaired under the same conditions. The action of ehtidium bromide on mitochondrial DNA appears to be completed within 18 hours of exposure to the drug. Total cellular macromolecular synthesis under ethidium bromide is initially decreased and at later times slightly stimulated. Ethidium bromide pretreatment of HeLa cells did not significantly affect the multiplication of Herpes simplex virus as compared with that in control cells.

Recently we reported the initial stimulation post infection (p.i.) of mitochondrial DNA synthesis in Herpes simplex virus (HSV) infected HeLa cells (1). Prerequisite for this effect is the multiplication of the infecting virus (K. Radsak and H. Freise, in preparation). It was therefore of interest to elucidate whether the information of the mitochondrial genome might be essential for the multiplication of the virus. Ethidium bromide (EB) has been shown to selectively inhibit mitochondrial DNA synthesis at suitable concentrations (2, 3). Under adaequate conditions it also destroys the tertiary structure of mitochondrial DNA (4, 5). This effect is irreversible within a certain time intervall after which mitochondrial DNA is restorated (6).

The purpose of this investigation was to establish by the use of EB a temporary condition in HeLa cells where nuclear

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macromolecular synthesis is essentially unimpaired and the expression of the mitochondrial genome is abolished. Such a system could serve as a tool to clarify a possible role of mitochondrial DNA in virus maturation.

In our experiments we determined (I) the minimal concentration of EB which inhibits mitochondrial DNA synthesis but does not interfere with nuclear nucleic acid and protein synthesis, (II) the minimal exposure time to this minimal concentration of EB that leads to depletion of mitochondrial DNA synthesis, (III) the kinetics of total cellular macromolecular synthesis under a minimal EB concentration as a parameter of nuclear nucleic acid and protein synthesis. HeLa cells in which mitochondrial nucleic acid synthesis was thus inhibited but nuclear nucleic acid synthesis was essentially unaffected were used for viral multiplication studies within 48 hours after removal of the drug (i.e. before repair of mitochondrial DNA started).

Materials and Methods Herpes simplex virus (Type 1) and HeLa cells from our laboratory stock were used in all experiments. Culture conditions and mode of infection were as described before (1).

Crude preparations of mitochondrial DNA were extracted from HeLa cells according to a modification of the method of Hirt (7, 1). Nuclear DNA was extracted from the pellet after resuspension in 0.01 M Tris HCl, 0.001 M EDTA buffer, pH 7.5 following the method of Thomas et al. (8). Isopycnic centrifugation in cesium chloride(CsCl)-EB solution according to Radloff et al. (9) was used for the final separation of covalently closed circular DNA of mitochondria in the crude preparation mentioned above from contaminating nuclear fragments. Centrifugations were performed in a 65 fixed angle rotor of a Beck-

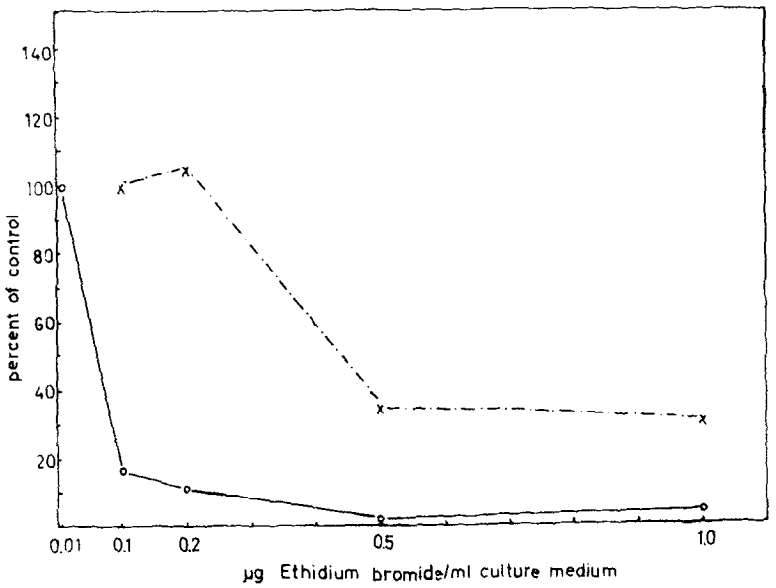

Fig. 1. Effect of EB on mitochondrial (o——o) and nuclear (x- - -x) DNA synthesis in HeLa cells. Different concentrations of EB were added simultaneously with 3-H-thymidine (5 µC/ml) to the culture medium of 5 x 10^6 HeLa cells for 42 hours. Mitochondrial and nuclear DNA synthesis was calculated as described in Results and compared with control samples. At a concentration of 0.2 µg EB/ml M-DNA was almost completely inhibited whereas nuclear DNA synthesis was unimpaired.

man L 2 centrifuge. Centrifugation conditions were as previously described (1).

DNA and protein were quantitated (1, 10) and radioactivity assayed (3) as described before. Infectious virus was titrated in tubes of primary rabbit kidney cells as reported previously (11).

Results

Effect of EB on mitochondrial and nuclear DNA synthesis:

EB was added to parallel cultures of 5 x 10^6 HeLa cells at various concentrations (Fig. 1) simultaneously with 3-H-thymidine (5 µC/ml). After 42 hours cells were harvested and a crude extract of mitochondrial DNA was separated from nuclear DNA using a modification of the method described by Hirt (7,

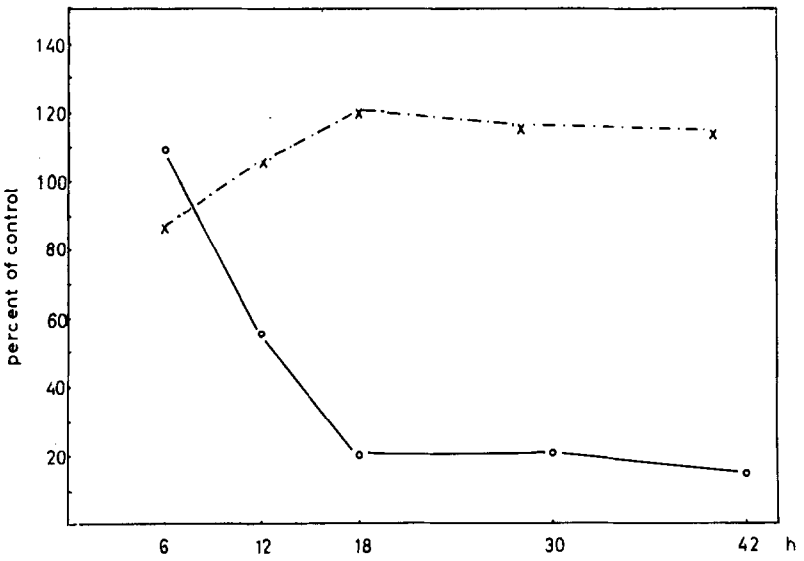

Fig. 2. Kinetics of the action of EB on mitochondrial (o——o) and nuclear (x-·-·-x) DNA. At zero-time 3-H-thymidine (5 µC/ml) was added to the culture medium of 5 x 10^6 HeLa cells for 42 hours. 6, 12, 18, 30 and 42 hours before harvesting the cells EB was added at a concentration of 0.2 µg/ml. M-DNA and nuclear DNA synthesis was calculated as described in Results and compared with control samples. M-DNA degradation seems to be completed after 18 hours of exposure to EB. Nuclear DNA synthesis has overcome the initial inhibition at the same time.

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1). Supercoiled DNA of mitochondrial origin was separated from linear double stranded contaminants by centrifugation in CsCl--EB solution (9). Total radioactivity in the position representative for supercoiled nicked or EB-altered mitochondrial DNA (12) was taken a parameter for mitochondrial DNA synthesis. Nuclear DNA synthesis was estimated from the specific radioactivity (cpm 3-H/µg DNA) of the DNA extracted from the 'Hirt--pellet' of the same sample. Fig. 1 shows that at a concentration of 0.2 µg EB/ml culture medium synthesis of mitochondrial DNA is inhibited by approximately 90 % whereas nuclear DNA synthesis is essentially unaffected.

Kinetics of the effect of EB on mitochondrial and nuclear DNA: To determine the minimal exposure time of HeLa cells to

EB that leads to disappearance of circular mitochondrial DNA we exposed parallel cultures of 5 x 10^6 HeLa cells to 0.2 µg EB/ml culture medium for various time intervalls (Fig. 2). Labelling with 3-H-thymidine was carried out continously for 42 hours. EB was added 6, 12, 18, 30 and 42 hours before terminating the experiment to one of the replicate cultures. This experimental schedule was chosen to follow the kinetics of EB-induced alteration of mitochondrial DNA. Isolation and characterization of mitochondrial and nuclear DNA and the evaluation of the results were the same as described above. Fig. 2 shows that the action of EB on mitochondrial DNA seems to be completed within 18 hours of exposure. In contrast to the progressively inhibitory effect of EB on mitochondrial DNA nuclear DNA synthesis as measured by the specific radioactivity is initially slightly inhibited and then continuously exceeding that of the control by 10 - 20 percent.

Effect of EB on total cellular DNA-, RNA- and protein synthesis: For the establishment of a cellular condition which lacks 'normal' mitochondrial DNA it was essential to examine nuclear RNA- and protein synthesis in addition to DNA synthesis under the minimal concentration of EB that suppresses mitochondrial DNA synthesis. It has been reported that mitochondrial RNA- and protein synthesis appear to be at least as sensitive to EB as DNA synthesis (13, 14, 15, 16).

Total cellular DNA-, RNA- and protein synthesis was measured by pulse labelling of 5 x 10^6 HeLa cells with 3-H-thymidine, 3-H-uridine and 3-H-leucine, respectively, for one hour prior to the times given in Fig. 3. Synthesis was estimated from the incorporation of cpm 3-H/µg cellular protein. Mitochondrial macromolecular synthesis can be neglected in this experiment

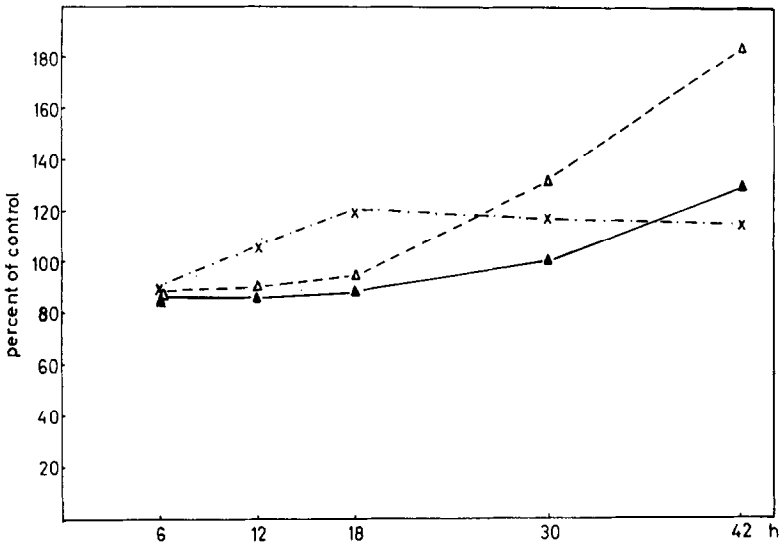

Fig. 3. Effect of EB on total cellular DNA (x—·—·—x), RNA (Δ- - -Δ), and protein (▲——▲) synthesis in HeLa cells. Puls labelling was carried out with 3-H-thymidine, 3-H-uridine and 3-H-leucine, respectively, (1 μC/ml) for 1 hour preceding the times given in the figure. Macromolecular synthesis was calculated from cpm 3-H-incorporated/μg cellular protein. The initial inhibition of cellular RNA and protein synthesis appears to last longer than that of DNA synthesis.

setup since it amounts to only 0.2 percent of cellular macromolecular synthesis (17).

Multiplication of Herpes simplex virus in EB-preteated HeLa cells: HeLa cells used for virus infection were pretreated for 20 - 24 hours with 0.2 μg EB/ml culture medium. Cell monolayers were then rinsed 3 - 4 times with buffered salt solution and kept consecutively under drugless medium for at least 2 hours to minimize the amount of cellbound drug and its possible photoinactivating effect on the virus. Replicate cultures of 5×10^6 cells were then infected with the virus at a multiplicity of about 1 - 3 infectious units/cell. The virus was added to the serumless culture medium of 10 ml. At definite times p.i. aliquots of the supernatant culture medium were removed and assayed for infectivity. This mode of infection was chosen to examine

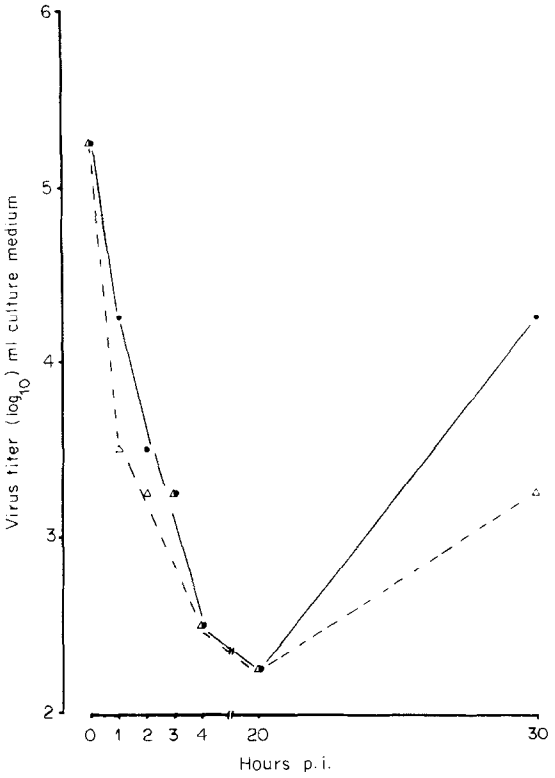

Fig. 4. Multiplication of Herpes simplex virus in HeLa cells pretreated with EB (0.2 µg/ml culture medium for 20 - 24 hours) (Δ---Δ) and in untreated (●——●) control cultures. Virus titers reached in pretreated cultures were slightly lower than those of the controls.

a possible difference in virus adsorption of EB-treated cells as compared with untreated control cultures. Fig. 4 shows the result of one of these experiments. A significant difference between experiment and control could neither be found with respect to the kinetics of virus adsorption nor in the final virus titers.

Discussion

Our results indicate that after an exposure time of 18 hours essentially no mitochondrial DNA can be recovered from EB--treated HeLa cells with the standard techniques described. When the ultrastructure of mitochondrial DNA was examined by

the Kleinschmidt technique there was evidence that the tertiary structure was destroyed (unpublished data). Mitochondrial RNA synthesis has been shown to be even more sensitive to the action of EB than DNA synthesis (14). We therefore assumed that HeLa cells kept under the conditions described lack a functioning mitochondrial genome. As shown before restoration of circular double stranded DNA does not start before 48 hours after removal of the drug (6) and is carried out probably by a special enzyme (12).

Multiplication of Herpes simplex virus in EB-pretreated cells did not differ significantly from that in control cells as examined by the yield of infectious virus. The small difference in the titers reached 30 hours after infection might be explained by the fact that EB-treatment leads to irreversible cell dammage of part of the cells which cannot take part in the production of infectious virus.

Stimulation of mitochondrial DNA synthesis at an early stage after infection of HeLa cells with Herpes simplex virus seems therefore not to be an essential prerequisite for virus multiplication.The finding that isolated mitochondria from HSV infected HeLa cells do not exhibit an increased DNA synthesis (K. Radsak, submitted for publication) suggests that the initial stimulation of mitochondrial DNA synthesis after HSV infection in infected cells seems to be an indirect consequence of the virus-induced alterations of the cell metabolism.

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REFERENCES

1. Radsak, K., and Freise, H., Life Sci. 11, Part II, 717 (1972).
2. Nass, M.K., Proc.Natl.Acad.Sci.U.S. 67, 1926 (1970).
3. Radsak, K., Kato, K., Sato, N., and Koprowski, H., Exp. Cell Res. 66, 410 (1971).
4. Nass, M.K., Tenth Annual Meeting of the American Society for Cell Biology, J.Cell Biol. 47, 147a (1970).
5. Koch, J., Eur.J.Biochem. 30, 53 (1972).
6. Kato, K., Radsak, K., and Koprowski, H., Z.Naturforschg. 27b, 989 (1972).
7. Hirt, B., J.Mol.Biol. 26, 365 (1967).
8. Thomas, C.A., Berns, K.I., and Kelly, T., Procedures of Nucleic Acid Res., Cantoni, G.L., and Davies, D.R., Eds., Harper and Row, Publ. New York and London, 535 (1966).
9. Radloff, R., Bauer, W., and Vinograd, J., Proc.Natl.Acad. Sci.U.S. 57, 1514 (1969).
10. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J., J.Biol.Chem. 193, 265 (1951).
11. Kohlhage, H., and Schieferstein, G., Arch.ges.Virusforschg. 12, 640 (1965).
12. Smith, Ch.A., Jordan, J.M., and Vinograd, J.Mol.Biol. 59, 255 (1971).
13. Zylber, E., and Penman, S., J.Mol.Biol. 44, 195 (1969a).
14. Zylber, E., and Penman, S., J.Mol.Biol. 46, 201 (1969b).
15. Coote, J.L., Work, T.S., Eur.J.Biochem. 23, 564 (1971).
16. Perlman, S., and Penman, S., Nature (Lond.) 227, 133 (1970).
17. Nass, M.K., Science 165, 25 (1969).